



TITLE:

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CHANGES IN SMOOTH MUSCLE CELL PHENOTYPE AND CONTRACTILE FUNCTION FOLLOWING ISCHEMIA-REPERFUSION INJURY IN THE RAT URINARY BLADDER

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Twenty-eight adult male Sprague-Dawley rats were divided into four groups: Group 1 received 1 hour (h) of bilateral ischemia alone. Groups 2 and 3 received 1 h ischemia followed by 1 and 4 h of reperfusion (I-R), respectively. Group 4 consisted of age-matched control rats. Bladder strips were studied using electrical field stimulation (EFS) and KCl stimulation. Maximal contractile responses were recorded and analyzed. Temporal patterns of changes in phenotypic (non-contractile and contractile) expression of bladder smooth muscle cells were investigated using electron microscopy. The mean ratio of non-contractile to contractile phenotype (nc/c) of smooth muscle cells (SMCs) in the control group was 0.169. In the *ischemia alone* group, the ratio was 0.991. In the 1 h I-R group, the ratio 0.865 whereas in 4 h I-R group the ratio 1.601. The contractile responses to EFS and KCl showed decreased responses in all groups. These results clearly demonstrated that the ratio of nc/c increased significantly in the ischemia group and further increased significantly in both I-R groups. The contractile responses decreased in all ischemic groups although the magnitude of the contractile changes did not correspond in the change of phenotype ratio.

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Key words: Phenotypic expression, Bladder smooth muscle cell, Ischemia-reperfusion injury, Rat

INTRODUCTION

In our clinical experience, voiding dysfunction is not always improved after removal of outlet obstruction of the bladder by surgical treatment in patients with benign prostate hypertrophy (BPH). In such patients, the bladder contractility and compliance in the cystometrogram findings are not improved. In these cases, irreversible morphological and functional changes might have occurred before the surgical treatment. However, it is difficult to predict the irreversible changes in the bladder function before surgery. In order to predict the changes described above and to improve the irreversible changes, it is important to understand the mechanisms of morphological changes occurring in the bladder wall after long-term bladder outlet obstruction (BOO)^{1,2)}. There is increasing evidence that ischemia and reperfusion (I-R) are major etiological factors in the progression of bladder dysfunction induced by partial BOO³⁾.

Recently, arterial smooth muscle cells (SMCs) including the carotid artery have been classified into contractile and synthetic phenotypes by morphological, biological and functional characteristics, and arterial phenotypic expression has been shown to be implicated in the initiation of atherogenesis^{4,5)}. Contractile phenotypes of SMCs have cytoplasm filled with myofilaments and synthetic organelles are scattered, and contraction is

a basic function of contractile phenotypes of SMCs. In contrast, the synthetic phenotypes of SMCs have cytoplasm developing organelles to produce and secrete various kinds of cytokines and extracellular matrix, and myofilaments in them are scattered; they are considered to be involved in the pathogenesis of blood vessels including aortic stenosis. We have previously shown that smooth muscle cells in the bladder can be classified into two phenotypes in a manner similar to vascular smooth muscle⁶⁻⁸⁾.

In the present study, we investigated the phenotypic changes of bladder SMCs in pathologic conditions by electron microscopic examination and correlated the magnitude of these changes with detrusor contractile changes in the rat urinary bladder.

MATERIALS AND METHODS

Twenty eight adult male Sprague-Dawley rats (CLEA, Tokyo, Japan), weighing 250–350 g were divided in the four groups: those receiving 1 hour (h) of ischemia alone (*Ischemia alone*), 1 h of ischemia followed by 1 h reperfusion (1 h I-R), and those followed by 4 h of reperfusion (4 h I-R). In addition, an age-matched control group (*control*) was evaluated. Experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals by the Japanese Pharmacological Society.

1) Ischemia-reperfusion-recovery model

The rat urinary bladders were subjected to I-R as described previously³⁾. Animals were anesthetized with

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ketamine/xylazine solution (80/10 mg/kg, intraperitoneal). Under sterile conditions, the bladder was exposed via a midline suprapubic incision and both of the vesical arteries were identified. *In vivo* ischemia was created by reversibly clamping the vesical arteries for 1 h using disposable vessel clips (TKM-1 type) with a holding force of 60 grams (BEAR Medic Corporation, Ichikawa-city, Chiba, Japan). Reperfusion was initiated by removal of the clamps from the bladder for either 1 and 4 h. Another group consisted of age-matched control rats. Immediately after the procedures, the rats were anesthetized, the bladder removed and cut in half, and then the animals were euthanized.

2) Bladder strip preparation and Contractile responses

After the bladder was removed and weighed, four longitudinal strips were obtained. Each strip was mounted in a separate 2 ml bath containing Tyrode's solution (124.9 mM NaCl, 2.6 mM KCl, 23.8 mM NaHCO₃, 0.5 mM MgCl₂, 0.4 mM NaH₂PO₄, 1.8 mM CaCl₂, and 5.5 mM dextrose) maintained at 37°C and equilibrated with a mixture of 95% O₂ and 5% CO₂. An initial resting tension of 0.5 grams was applied for 30 minutes, and responses were recorded isometrically using a force-displacement transducer (UL-10-GR, Minebea Co. Ltd, Tokyo, Japan). The muscle strips were allowed to equilibrate for at least 1 h before the experiments were begun. Electrical field stimulation (EFS) was applied through 2 parallel platinum electrodes, with 0.3-msec square wave pulses being applied for periods of 10 seconds separated by 5-minute intervals, except as otherwise stated, and supramaximal voltages (30 volts) at 2, 8 and 32 Hz. were employed. Following EFS, the maximal responses were determined sequentially for KCl (120 mM). A series of 3 washes, at 15-min intervals, with Tyrode's solution followed each of the pharmacological stimulations.

3) Electron microscopic observations

Each bladder specimen sectioned transversely was immersed in 4% glutaraldehyde in 0.1 M phosphate buffer and, following fixation, trimmed into 1 to 2 mm small cubes and postfixed in 1% aqueous osmium tetroxide for 2 h. Specimens were then dehydrated and embedded in Epon-812 resin (TAAB Laboratories, Berkshire, UK). Semithin (1mm thick) sections from each block were stained with toluidine blue (Aldrich, Los Angeles, CA, USA) and examined by light microscopy to select the most appropriate blocks for thin sectioning. Thin sections were cut, mounted on uncoated copper grids, double stained with uranyl acetate (Wako Chemicals, Neuss, Germany) followed by lead nitrate (TAAB, UK) and examined in a Hitachi-7100, H-800 transmission electron microscope (HITACHI, Hitachinaka, Japan). Eight photographs were taken from each of 5 blocks. The number of cells of contractile phenotype and synthetic phenotype which were recognized according to their morphological criteria was counted in one photograph and the ratio of

synthetic and contractile phenotype (nc/c) was calculated.

The recognition of synthetic and contractile phenotype was based upon the morphological criteria suggested by Mosse et al with slight modification⁴⁾. Briefly, 1) Synthetic type: Synthetic organelles including granular endoplasmic reticulum and Golgi apparatus are rich but myofilaments and basilemma are not present; 2) Intermediate type: Myofilaments are scattered but cytoplasm is filled with intracellular organelles around nucleus; 3) Contractile type: Cytoplasm is filled with myofilaments, and dense body and basilemma are present, but other organelles are scattered. In the present study, phenotype was classified into two types, non-contractile type (synthetic type+intermediate type) and contractile type of SMCs.

4) Data analysis

Data are shown as mean \pm standard error of mean (SEM). Comparisons among groups were made using the analysis of variance followed by the Neuman-Keuls test for individual differences with p value <0.05 considered significant.

RESULTS

1) Temporal pattern of changes in bladder mass after bladder outlet obstruction

The mean bladder weights for control, *ischemia alone*, 1 h I-R and 4 h I-R were 149.51 ± 8.57 , 151.36 ± 4.25 , 134.66 ± 6.11 , and 156.59 ± 9.51 mg, respectively. There were no significant differences in bladder weight among the groups.

2) Phenotypic changes in bladder smooth muscle cells

Fig. 1 shows representative electron micrographs of tissues from control (A), Ischemic (B), 1 h I/R (C), and 4 h I/R (D). The ratio of nc/c is presented in Fig. 2. The ratio of non-contractile to contractile phenotype (nc/c) of SMCs in the control group was 0.169 ± 0.033 . In the *ischemia alone* group, the ratio of nc/c of SMCs was increased significantly to 0.991 ± 0.268 compared with the control group. The ratio of nc/c in the 1 h I/R was similar to the *ischemia alone* at 0.865 ± 0.167 whereas in the group of 4 h I-R the ratio of nc/c increased significantly to 1.601 ± 0.265 .

3) Measurement of contractile responses

The results for maximal tension generated to EFS and KCl are shown in Fig. 3. The maximal responses to all frequencies of EFS were reduced below the control in all ischemia and I-R groups, and significantly in the 1 h I-R group. The responses among the ischemic and ischemia followed by reperfusion groups were generally similar to each other showing no significant differences among the ischemic and I/R groups.

DISCUSSION

The structure, function, molecular and cell biological characteristics of the smooth muscle cells are variable under normal and pathological conditions in the blood

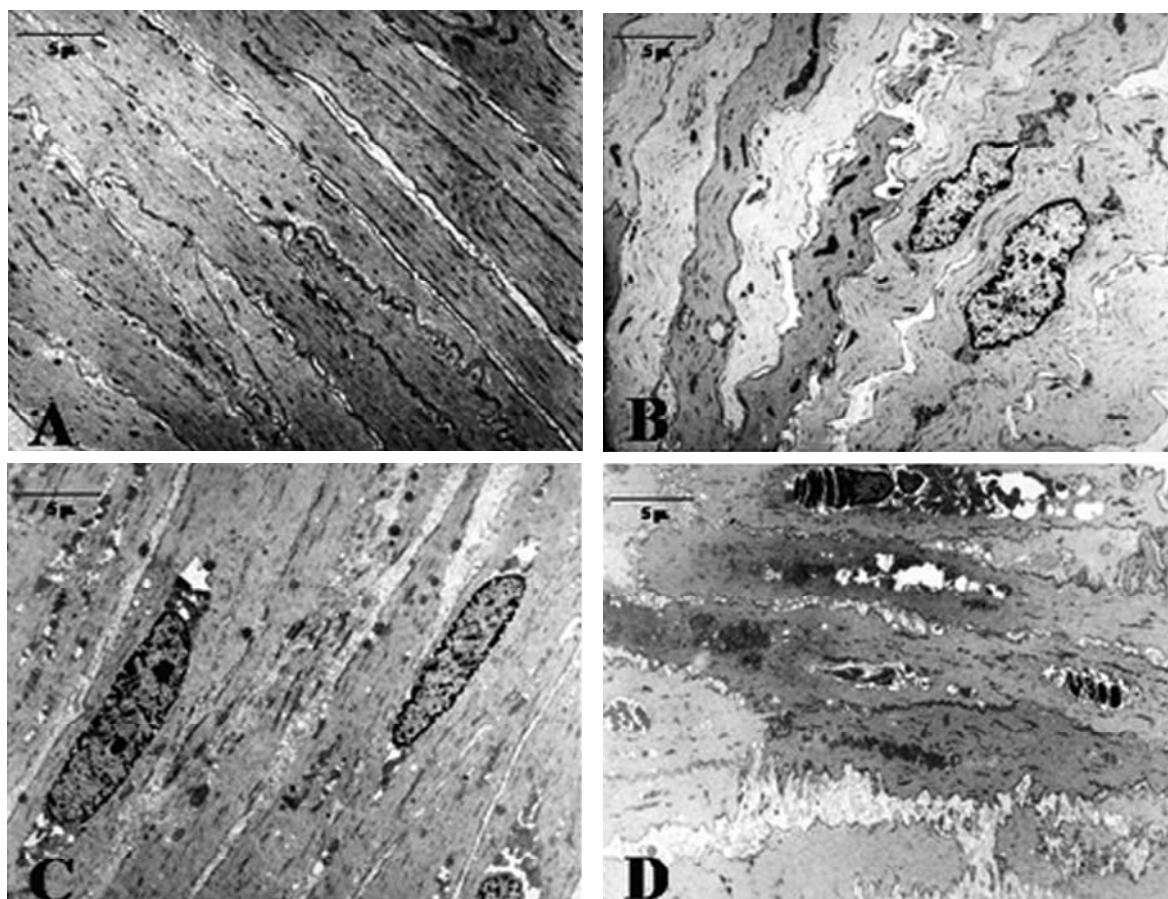


Fig. 1. Electron micrographs of smooth muscle cells obtained from rat urinary bladder on ischemia-reperfusion injury. A: age-matched control, $\times 2,000$. Contractile phenotype; Myofilaments and dense bodies are observed but synthetic organelles are scattered. B: 1 h of ischemia alone, $\times 2,000$. C: 1 h of ischemia followed by 1 h of reperfusion, $\times 2,000$. D: 1 h of ischemia followed by 4 h of reperfusion, $\times 2,000$. Number of synthetic phenotype is increased. (measure of bar: 5μ).

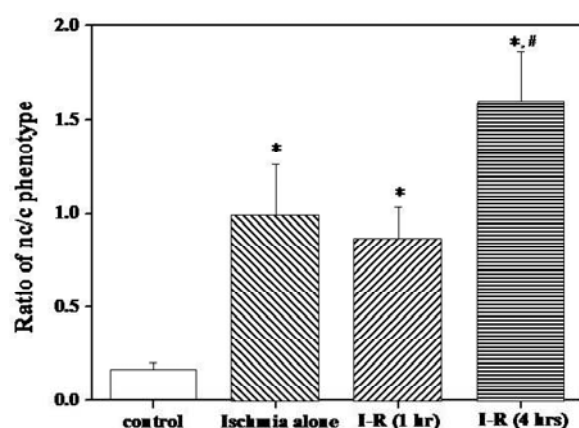


Fig. 2. Phenotypic changes in bladder smooth muscle. nc/c represents the ratio of non-contractile (synthetic) to contractile phenotype in the field of one electron micrograph obtained from bladder smooth muscle. Data is shown as mean \pm standard error of mean. * significant difference from age-matched control group ($p < 0.05$). # significant difference from 1 h I-R group ($p < 0.05$).

vessels^{4,5}). The transformation of SMCs from contractile to synthetic phenotype is observed in pathological

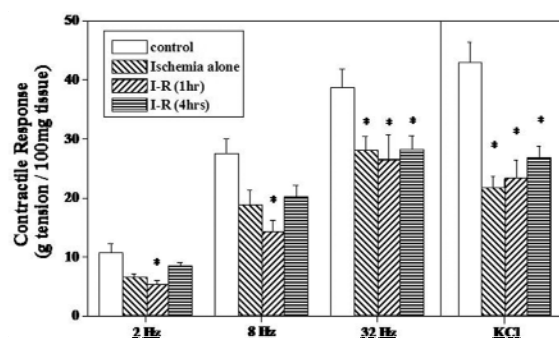


Fig. 3. Effect of ischemia and reperfusion on the *in vitro* response of the contractile response to EFS (2, 8 and 32 Hz) and KCl (120 mM). Data is shown as mean \pm standard error of mean. * significant difference from age-matched control group ($p < 0.05$).

conditions. These transformed cells express characteristic cytoskeleton protein, contractile protein and receptor protein. The studies on these transformed cells have been conducted using the blood vessels^{4,5} and cultured SMCs⁹). In the SMCs of blood vessels, the non-contractile phenotype (mainly synthetic type) is increased in abnormal conditions. With the BOO

model in rat urinary bladder using partial BOO, we previously found that contractile and synthetic phenotype expression were observed in bladder SMCs as reported in vascular SMCs and the remarkable increase in the ratio of nc/c phenotype correlates with the deterioration of the bladder function⁶⁻⁸⁾. We have reported phenotypic changes with aging and bladder diseases in human bladder SMCs⁷⁾. The ratio of contractile phenotype was especially decreased with neurogenic bladder and the ratio of nc/c phenotype individually decreased with age. We considered that bladder disease caused a conversion of contractile into synthetic or intermediate phenotype, and this modulation of phenotype of SMCs may play an important role in bladder contractile dysfunction.

Lau et al. classified cultured bladder SMCs into long and short cells by microscopical examination¹⁰⁾. Whether their long and short cells coincide with the contractile and non-contractile phenotype, respectively, in our study is uncertain. In cultured bladder SMCs, morphological classification was conducted and functional difference of these cells was suspected by the expression of contractile proteins. The long and short cells may be considered to coincide with the contractile and non-contractile (synthetic) phenotypes, respectively. Baskin et al investigated the morphological changes in human and bovine fetus bladder SMCs, and reported that there are morphological differences between the bladder SMCs of human and bovine fetus¹¹⁾. These changes could be distinguished by classifying the phenotype of bladder SMCs. Elbadawi et al investigated the morphological changes in the electron micrographs of human bladder wall after BOO, and reported that there are various changes in the intermediate cell junction and extracellular space, but they did not mention whether these changes are due to the phenotypic changes or transformation of bladder SMCs¹⁾. We considered that changes in the bladder wall are caused by transformation of the bladder SMCs in the context of phenotypic change, and that these changes might alter the bladder function. Gosling et al reported that hypertrophy was a significant characteristic change observed at 3 to 7 days after BOO, and that there were no significant ultrastructural changes in the cells by electron microscopic examination²⁾. They found the indistinct myofilaments and irregularly clumped sarcoplasmic dense body at 18 days, and swelling of mitochondria in all cells of bladder wall at 70 days after the obstruction. Taking into consideration our phenotypic concept and the results of their rabbit experiment, the contractile phenotype may be dominantly expressed at 3 to 7 days, and non-contractile (synthetic) phenotype may be dominantly expressed at 70 days after the obstruction. These results might coincide with our previous data⁶⁾. They also investigated the contractility of the bladder smooth muscle by electrical field stimulation, and observed a

decrease in the contractility of bladder smooth muscle after long-term obstruction.

We previously reported that short time I/R leads to bladder dysfunction as well as to the induction of lipid peroxidation¹²⁾. Both bladder dysfunction and the induction of lipid peroxidation are prevented by antioxidants (free radical scavengers) and other neuroprotective medications may have therapeutic benefit in patients with bladder outlet obstruction as well as older adults with acute and possibly chronic urinary retention. In the present study, we correlated the morphological and functional changes in rat urinary bladder SMCs following I-R injury. There was a significant 6-fold increase in the ratio of nc/c cells between control and ischemia groups. The ratio of the 1 h I/R increased similarly; but by 4 h reperfusion, the ratio increased significantly by two-fold over the ischemia group and the 1 h I/R group. In the increased ratio of nc/c in the ischemia group and 1 h I/R group correlated with a 30–50% decrease in contractile responses as was expected. However, there was a second significant increase in the ratio of nc/c between the 1 and 4 h reperfusion groups which was not correlated with an increased level of contractile dysfunction. This would indicate that the contractile cells remaining can compensate for the increased the ratio of nc/c phenotype; at least for a short time; and that the change in the ratio of nc/c can precede changes in contractility. Interestingly, in the model of the I-R injury on the rat urinary bladder, the ratio of nc/c in the group of 4 h I-R was at approximately the same level at 10 weeks after bladder outlet obstruction⁶⁾. The bladder blood supply may play a major role in the morphological and functional changes from these in bladder dysfunction after BOO as already reported, and these damages may be influenced by the oxidative stress.

We previously reported that the marked increase in the ratio of nc/c phenotype correlates with the deterioration of the bladder function in the BOO model in rat urinary bladder. In the present study with the I-R model in rat urinary bladder, contractile and non-contractile (mainly synthetic) phenotypic expression were observed in bladder SMCs, and the results were classified by the ratio of nc/c phenotype. Furthermore, concerning the relationship with the bladder function, the study showed that there are pathological and time differences compared with this phenotype, giving such relationships potential to explain the background to the compensatory triggering of the bladder function.

CONCLUSION

Our findings demonstrate that the bladder SMCs nc/c phenotype ratio is significantly affected by both ischemia and ischemia followed by reperfusion. These data support the conclusion that the ratio of nc/c phenotype of bladder SMCs plays an important role in modulating bladder function.

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和文抄録

ラット膀胱虚血・再還流モデルにおける膀胱平滑筋細胞の
形態学的 Phenotype と膀胱機能の検討

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目的：下部尿路閉塞による膀胱機能低下には膀胱虚血・再還流が一要因であるという報告が増加している。今回、ラット膀胱虚血・再還流モデルを用いて膀胱平滑筋細胞における形態学的 phenotype の変化と膀胱機能の関係を検討した。

方法：28匹のSD雄ラットを用いて膀胱虚血・再還流（I-R）モデルを作製し、1時間虚血単独群、1時間虚血・1時間再還流群、1時間虚血・4時間再還流群、コントロール群に分類し、透過型電子顕微鏡による膀胱平滑筋細胞における phenotype の比率および経壁電気刺激とKClに対する等尺性収縮力を測定し、検討を行った。

結果：コントロールにおける膀胱平滑筋細胞の収縮

型と非収縮型の比率（nc/c比）は0.169、虚血単独群はnc/c比は0.991、1時間I-R群、4時間I-Rのnc/c比はそれぞれ0.865、1.601であった。経壁電気刺激とKClに対する等尺性収縮力はI-R群で減少していた。

考察：膀胱平滑筋細胞のnc/c比の結果より虚血単独群でも、非収縮型細胞が増加し、I-Rによりさらに非収縮型細胞が増加することが示された。膀胱平滑筋収縮力は虚血単独群でもI-R群でも減少したが、収縮反応は膀胱平滑筋細胞の phenotype の変化とは対応しなかった。

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